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Complete Host Range Testing on Common Reed with Potential Biological Control Agents and Investigation into Biological Control for Flowering Rush

Patrick Häfliger and Harriet L. Hinz

July 2016



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Complete Host Range Testing on Common Reed with Potential Biological Control Agents and Investigation into Biological Control for Flowering Rush

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Abstract

The noctuid moths, *Archanara geminipuncta* and *A. neurica*, were selected as the most promising candidates for biological control of common reed. Successful larval development was only found on *Phragmites* spp., but development was also possible on the native North American subspecies *P. australis* subsp. *americanus*. However, open-field oviposition tests showed a strong preference of females for both European and introduced *P. australis*. Because of the higher egg mortality on native reed, the authors expect any impact of *A. neurica* and *A. geminipuncta* on the native reed *P. australis* subsp. *americanus* to be negligible, should the noctuids be released in North America.

A biological control project for flowering rush was started in spring 2013. According to the authors' literature survey, the semi-aquatic weevil, *Bagous nodulosus*, is the most promising potential agent for biological control. During various field surveys in Northern Germany, the Czech and Slovak Republics, Hungary, Poland, and Serbia, the authors collected several hundred adults of *B. nodulosus*, established a rearing colony, and started with sequential no-choice oviposition tests. So far, no eggs were found on any of the 22 test plants offered. The authors also found the other five herbivore species recorded as monophagous on flowering rush and started work with a second weevil, *B. validus*.

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Preface

This report was prepared by Drs. Patrick Häfliger and Hariet Hinz, Centre for Agriculture and Bioscience International (CABI), Delémont, Switzerland, for the U.S. Army Engineer Research and Development Center, Environmental Laboratory (ERDC-EL). At the time of publication, Dr. Alfred F. Cofrancesco, CEERD-EZT, was the technical director for Environmental Engineering and Sciences-Civil Works; Dr. Linda Nelson, CEERD-EZT, was program manager for the Aquatic Plant Control Research Program. The Director for the Environmental Laboratory was Dr. Beth Fleming.

The work reported herein was performed by CABI Switzerland (Hariet Hinz and Patrick Häfliger). The following students assisted in the field and in the laboratory: R. Leiner, S. Soukou, C. Baan, A. Martins, D. Sjolie, M. Montoro Caceres, E. Smith, R. Maclean, and L. Streiff. Dr. I. Toševski (Institute for Plant Protection and Environment, Zemun, Serbia) performed the molecular analysis of the weevil, *Bagous nodulosus*, and was also involved in field work in Serbia.

The authors thank Henrich Klugkist (Senator für Umwelt, Bau und Verkehr, Bremen, Germany) and Karin Menke (Büro für Landschaftsökologie, Bremen, Germany) for supporting the permit to access the Landschaftsschutzgebiet Niedervieland and to collect *Bagous nodulosus*. The authors are also grateful to Otto Merkl, Attila Podlussány and Gábor Hegyessy for sharing site records for *Bagous validus*. Prof. M. von Tschirnhaus (Bielefeld, Germany) kindly provided information about *Phytoliriomyza ornata* and sent the team's flies to the Diptera specialist Jens-Hermann Stuke. Many thanks to Jens-Hermann Stuke (Leer, Germany), who examined the *Hydrellia* species. Martin Hanzl (Academy of Science of the Czech Republic) sent information about the ploidy level of *Butomus* populations from the Slovak Republic and the coordinates of their sites. The Teichwirtschaft Götsch in Muxall, Germany, kindly allowed the team access to their ponds. Petr Bogusch (University of Hradec Králové) assisted on a field trip to the Czech and Slovak Republics and provided site information and access permits. The authors also thank Rafal Gosik (Maria Curie-Sklodowska University, Lublin, Poland), for providing the coordinates of a *Bagous* site in Poland. They are grateful to

John Gaskin (USDA-ARS Sidney, Montana) who is conducting the molecular work on *Butomus* populations sampled in Europe and North America. Many thanks also to Jenifer Parson (Washington Department of Ecology) and Peter Rice (University of Montana, Missoula), who organized and shipped test plants. Florence Willemin (CABI Switzerland) propagated and maintained common reed, flowering rush and test plants. Tim Haye (CABI Switzerland) also took some great pictures of adult *B. nodulosus*.

In 2014 and 2015, the projects were financed by the U.S. Fish and Wildlife Service and State of New York Department of Environmental Conservation through Cornell University, the Washington Department of Agriculture, the Washington Department of Ecology, the Washington Department of Natural Resources, the Montana Weed Trust Fund through the University of Montana, and the U.S. Army Corps of Engineers in the USA, and the British Columbia Ministry of Forests, Lands and Natural Resource Operations in Canada.

At the time of publication of this report, COL Bryan S. Green was the Commander of ERDC, and Dr. Jeffery P. Holland was the ERDC Director.

1 Biological control of common reed, *Phragmites australis*

1.1 Open-field oviposition tests with *A. geminipuncta* and *A. neurica*

1.1.1 Background

In 1998, a project was started at CABI in Switzerland to evaluate the potential for biological control of common reed in North America. During a two-year survey, 15 sites in Central Europe were sampled for endophagous herbivores of *P. australis*. In a first step, eight moth species and one chloropid fly were prioritized for further investigations as potential biological control agents (Häfliger et al. 2001). Currently, work is focused on the two noctuid moths with the highest impact on common reed, *A. geminipuncta* and *A. neurica*.

Common reed, *Phragmites australis* (Cav.) Trin. ex Steudel, is a cosmopolitan, perennial, clonal grass that can form large, nearly monospecific stands in wetlands and along rivers and lakesides. Due to its high genetic and morphological variability, *P. australis* is able to grow in a wide range of habitats with different climates (van der Toorn 1972). In Europe, reed beds are inhabited by a rich insect community and are valuable and endangered ecosystems (Tscharntke 1999; Tewksbury et al. 2002). In North America and Australia, however, *P. australis* is considered invasive and a threat to biodiversity (Wapshere 1990; Marks et al. 1994; Tewksbury et al. 2002).

Only in the last century did *P. australis* start to spread in North America. Before that, it had been present for at least 3,500 years without being invasive (Orson et al. 1987). The dramatic increase of common reed populations in the second half of the 20th century has often been attributed to land use changes and eutrophication. However, the alternative hypothesis of the introduction of an invasive European genotype was verified by genetic studies of Saltonstall (2002). We now know that there are several native haplotypes in North America, but particularly in the East and Midwest they are usually rare and out-competed by the invasive European haplotype M (subsequently referred to as introduced reed). The native North American populations of common reed have been recognized as a distinct subspecies, *P. australis* subsp. *americanus* by Saltonstall et al.

(2004) (subsequently referred to as native reed). In addition, another lineage, *P. australis* subsp. *berlandieri* (E. Fourn.) Saltonst. & Hauber, was described from the U.S. Gulf Coast, (Saltonstall and Hauber 2007) (subsequently referred to as haplotype I). Lambertini et al. (2012) assume haplotype I to be a hybrid between *P. mauritanus* and an African/Mediterranean population of *P. australis*. Their data indicate haplotype I to be an “ancient introduction.” However, it is commonly considered as native (Gucker 2008; Ward and Jacono 2009).

For the development of biological control, the presence of native subspecies means that herbivores are required that can selectively reduce the invasiveness of the introduced European type without adversely affecting the native North American subspecies. Host-specificity tests carried out at CABI and at the University of Rhode Island demonstrated that both *Archana* species have a very narrow host range. Larvae were only able to complete development on plants in the genus *Phragmites*. However, larval development tests carried out in 2004 showed similar development rates on the native *P. australis* subsp. *americanus* and European *P. australis* (Häfliger et al. 2005).

An oviposition test carried out at CABI in 2013 showed a strong preference of *A. neurica* for reed populations collected in Europe and introduced reed collected in North America. Only 2.6% of the eggs were laid on native reed. In 2014, an additional experiment was conducted to confirm this finding for *A. geminipuncta*. In 2015, haplotype I was added in a similar experiment.

1.1.2 Objectives

The objectives of this research were to test the oviposition preference of *Archana geminipuncta* and *A. neurica* under open-field conditions for invasive vs. native reed and to maintain the rearing colonies of the two noctuid moths.

1.1.3 Approach

Adult moths used in these experiments were reared as follows: in mid-April, newly hatching larvae were transferred into cut stem pieces of common reed. Between 6 and 12 young shoot sections each containing two larvae of *A. geminipuncta* (only one larva for *A. neurica*) were placed onto moist horticultural sponge blocs in transparent plastic cylinders (37 cm high, 11 cm diameter) covered with a gauze lid. Cylinders were checked

daily and new shoot sections were offered as soon as a larva had left its shoot. Pupae were removed from stems, sexed, and up to five pupae were placed on a layer of vermiculite in a plastic cup (diameter 5.5-6.5 cm, height 8 cm) together with a wet cotton pad until adult emergence. One to three pairs of newly emerged moths were held for mating for one night in a wooden cage (40 x 40 x 65 cm) before release (Table 1).

Table 1. Origin of plants used in the open-field oviposition tests with *Archanara geminipuncta* and *A. neurica* in 2014 and 2015.

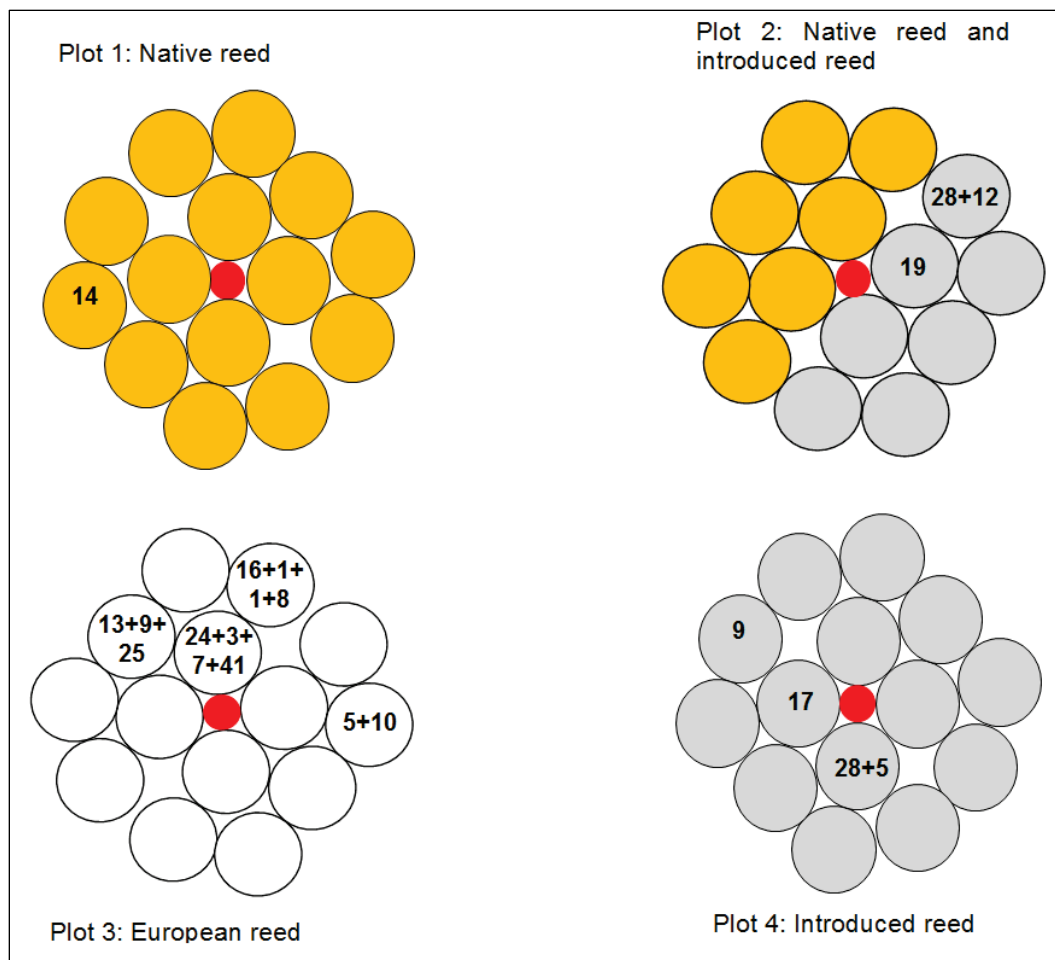
Population	Origin	Number of pots 2014	Number of pots 2015
Astoria, OR	Native	2	2
Beldens Landing, CA	Native	2	
Montezuma, NY	Native	3	3
Saratoga Springs, UT	Native	5	
Savage Fen, MN	Native	4	2
Seminary Fen, MN	Native	1	3
Spring Bluff, IL	Native	1	1
Sun Lake Park, WA	Native	3	1
Hillsborough, Canada	Native		2
Florida	Haplotype I		14
Assunpink Lake, NJ	Introduced	3	
Cape May, NJ	Introduced	4	1
New Haven, CT	Introduced	5	
Novato, CA	Introduced	4	1
Rock Ford, WA	Introduced	1	4
Saratoga Springs, UT	Introduced	2	2
MN	Introduced	2	6
Delémont, Switzerland	European	4	3
Magadino, Switzerland	European	2	1
Yverdon, Switzerland	European	2	2
Hodmezovasarhely, Hungary	European	3	
Krautsand, Germany	European		1
Iasi, Romania	European	3	2
UK	European		1
Serbia	European		4

Single open-field oviposition experiments were carried out in 2014 and 2015 on a meadow close to CABI in Switzerland (Figure 1). In 2014, four plots were set up as follows: 1) 14 pots of native reed; 2) 7 pots of native reed and 7 pots of introduced reed; 3) 14 pots of European reed; and 4) 14 pots of introduced reed (Figure 2). In 2015, the mixed plot (7 native/ 7 introduced) was replaced by 14 pots of haplotype I (Gulf Coast lineage) (Figure 3). Between five and eight different populations of reed per origin were randomly arranged in the plots, except for haplotype I in 2015, where only one population from Florida was available (Table 1). Each plot was placed in the corner of a 10x10 m quadrat (Figure 1) and mated pairs of noctuid moths were released in the center of each plot. In 2014 (between 14 and 24 July), 11 pairs of *A. geminipuncta* were released per plot; in 2015, 13 pairs of *A. neurica* (between 21 and 27 June) and 7 pairs of *A. geminipuncta* (between 9 and 18 July). Pots contained, at the time of insect release, on average between 9 and 22 stems, and mean shoot length was between 80 and 120 cm. In early August, all stems were harvested and checked for eggs.

Figure 1. Setup of the open-field oviposition test.



Figure 2. Setup and results of open-field oviposition test with *A. geminipuncta* in 2014 (red dot = moth release points; numbers indicate number of eggs laid on individual shoots).



1.1.4 Results

In 2014, 326 *A. geminipuncta* eggs were found on the exposed shoots (Table 2). Of these, 95.7% were laid onto European and introduced reed and only 4.3% on native reed. In 2015, *A. geminipuncta* only laid a total of 62 eggs. A similar proportion of these was laid on European and introduced reed and on haplotype I. A similarly low number of eggs was laid on native reed as in 2014. In contrast, *A. neurica* laid 441 eggs in 2015 (Table 3). Of these, 79.6% were laid on European and introduced reed, 7.5% on native reed, and 12.9% on haplotype I.

Figure 3. Setup and results of open-field oviposition test with *A. neurica* and *A. geminipuncta* in 2015 (red dot = moth release points; numbers indicate total number of eggs found on plant, black = *A. neurica*, red = *A. geminipuncta*).

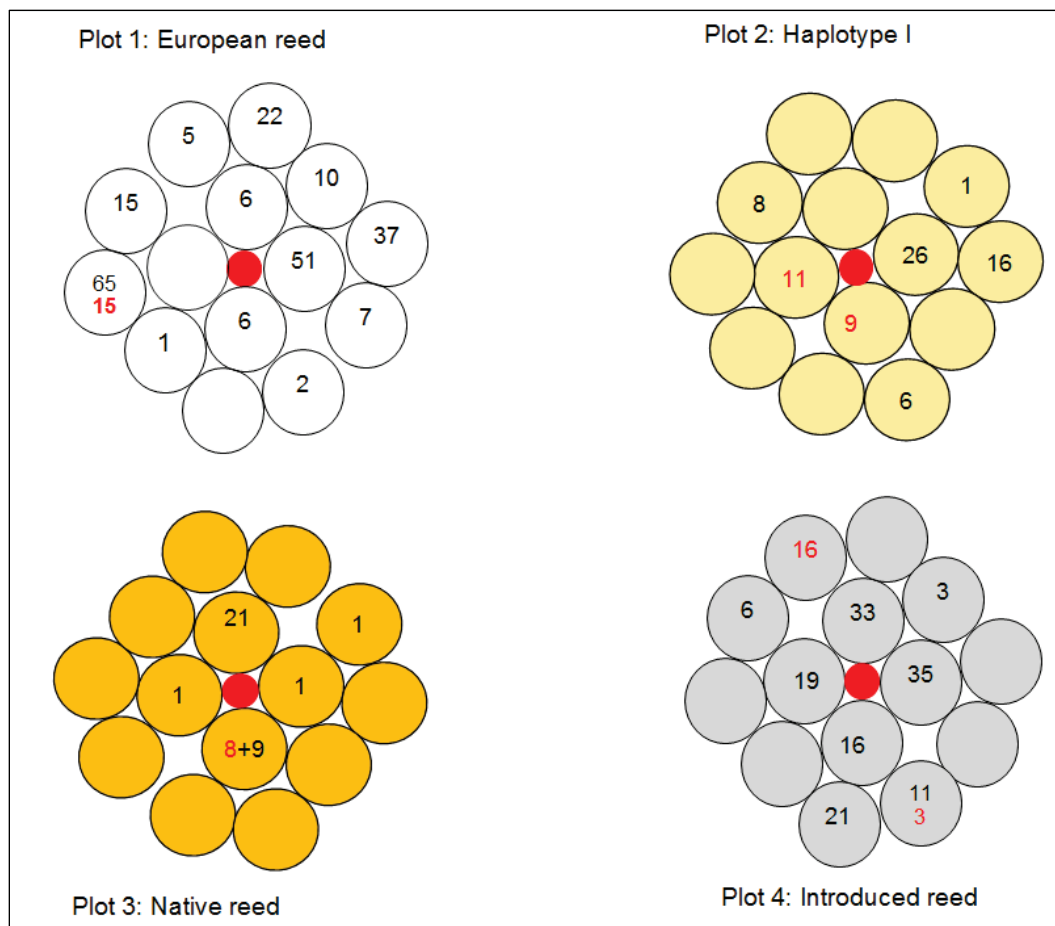


Table 2. Results of open-field oviposition test with *Archanara geminipuncta* in 2014.

Origin	Mean # stems per pot	Mean stem length (cm)	Mean stem diameter (mm)	# egg batches / # eggs
European ^a	22.0	116.7	3.4	16/194
Introduced ^a	18.2	98.4	3.5	7/118
Native ^b	15.4	122.7	4.1	1/14 ^c

^a *Phragmites australis*; ^b *P. australis* subsp. *americanus*

^c Two additional egg batches were found with a total of 39 eggs. However, these eggs were excluded, because they were laid on shoots that showed obvious characteristics of European/introduced reed and must have entered the pot via rhizomes that had grown in from neighboring plants.

Table 3. Results of open-field oviposition test with *Archanara neurica* (A.n.) and *A. geminipuncta* (A.g.) in 2015.

Origin	Mean # stems per pot	Mean stem length (cm)	Mean stem diameter (mm)	# egg batches / # eggs A.n.	# egg batches / # eggs A.g.
European ^a	22.6	79.6	3.3	34/207	3/15
Introduced ^a	11.5	82.1	3.8	26/144	2/19
Native ^b	9.6	119.3	4.9	7/33	1/8
Haplotype I ^c	15	48.3	5.0	10/57	2/20

^a *Phragmites australis*; ^b *P. australis* subsp. *americanus*; ^c *P. australis* subsp. *berlandieri*

1.1.5 Discussion and Conclusions

Previous no-choice larval development and oviposition tests with *A. geminipuncta* showed only minor differences in suitability between the native and the introduced subspecies of *P. australis*. However, the data presented here and a test carried out in 2013 (Häfliger, unpublished data) clearly show that both moth species strongly prefer invasive and European reed over native reed for oviposition when given the choice in an open field setting. Leaf sheaths of native reed tend to fall off before winter while leaf sheaths of introduced reed stay on the plant over winter. The team's results indicate that ovipositing females are able to somehow notice the difference and avoid the stems of native reed.

The fact that *A. geminipuncta* laid five-fold fewer eggs in 2015 compared to 2014 is potentially due to the fact that *A. neurica* adults were released first and that most of the best leaf-sheaths for oviposition may have been already occupied by eggs of *A. neurica*. Although fewer females were released of *A. geminipuncta* than *A. neurica*, the team would have expected to find at least three times more *A. geminipuncta* eggs based on previous experience.

In 2015, haplotype I was used exclusively in one of the test plots rather than a combination of native and introduced reed. This subspecies of *P. australis* does not lose its leaf sheaths during winter. It is not winter hardy, but stems usually do not completely die off during winter and often develop many side shoots in the following year. Overall, both *A. neurica* and *A. geminipuncta* laid approximately twice as many eggs on haplotype I than on native reed. Since stems of haplotype I are often older than one year and therefore much harder, this could theoretically limit larval

development of both moths. This is currently being investigated in the quarantine facility of the University of Rhode Island.

In addition, an overwintering experiment carried out in a common garden in 2006/2007 suggested that any eggs laid on native reed would suffer higher mortality due to differences in phenology of the two *P. australis* subspecies (Häfliger and Foresti 2008). Eggs laid on native reed will likely fall off together with leaf sheaths before or during winter and thus be exposed to climatic conditions, predators, and pathogens. Eggs overwintering unprotected on the soil had a 42% higher mortality ($P = 0.008$) compared to eggs protected under leaf sheaths (Häfliger and Foresti 2008). The team expects mortality of unprotected eggs to be even higher under field conditions. Especially on reed inundated by water, eggs might get washed away, or changing moisture regimes may increase fungal attack. Should the noctuids be released in North America, their impact on native reed is expected to be negligible due to low egg production, and higher egg mortality due to plant phenology. The authors are currently contributing to the petition for field release of *A. geminipuncta* and *A. neurica*, which is being prepared by Richard Casagrande (University of Rhode Island) and Bernd Blossey (Cornell University).

2 Investigation into biological control of flowering rush, *Butomus umbellatus*

2.1 Background

Flowering rush (*Butomus umbellatus* L.) is a perennial aquatic plant that grows along lake shores and in slow-moving bodies of water, irrigation ditches, and wetlands in temperate Europe and Asia. In several European countries, the plant is considered rare and endangered (Stöhr et al. 2006; Raabe et al. 2011). Fluctuating water levels favor the plant. It usually grows as an emergent with upright foliage in up to 60 to 80 cm deep water (Hroudová 1989). In North America, where *B. umbellatus* was introduced as an ornamental more than 100 years ago, the common emergent form is found in up to 3 m deep water. Submerged populations with flexible leaves suspended in the water column can be found in up to 6 m deep water (Jacobs et al. 2011). Flowering rush is now considered an aggressive invader of freshwater systems, and is becoming an increasing problem in the midwestern and western states of the USA and western Canada.

Two ploidy levels are known for *B. umbellatus*: diploids ($2n = 26$) and triploids ($2n = 39$). Plants of the two ploidy levels differ in various ways. Diploids produce abundant fertile seeds, whereas triploids produce far fewer and sterile seeds (Krahulcová and Jarolímová 1993). In Europe, low seed fertility in triploids is compensated for by production of bulbils (vegetative reproductive structures) in flower heads and increased production of lateral rhizome buds (Hroudová and Zákavský 1993), while in North America, bulbils in flower heads have only been found in diploids (Kliber and Eckert 2005). Despite heavy investment in seed production by diploids, little or no evidence of sexual recruitment was found in North America, suggesting predominantly clonal reproduction via bulbils (Fernando and Cass 1997; Kliber and Eckert 2005; Lui et al. 2005). In contrast, North American triploids invest heavily in a large, carbohydrate-rich rhizome and appear to only propagate by rhizome fragmentation (Thompson and Eckert 2004; Brown and Eckert 2005). Rhizome fragments, broken at fine constrictions by minor disturbances such as moving water, waves, and passing boats or waterfowl, disperse on water currents, sometimes over long distances (Jacobs et al. 2011). Sparsely

vegetated or unvegetated silty substrate, where water is shallow and currents have slowed, are ideal for establishment (Jacobs et al. 2011).

Since no effective long-term control methods are currently available, a biological control project was started in spring 2013 on the initiative of Jennifer Andreas (Integrated Weed Control Project, Washington State University), and CABI in Switzerland was subcontracted to conduct surveys for potential insect agents. After a literature survey, four herbivores were prioritized as the most promising potential biological control agents; the weevils *Bagous nodulosus* and *B. validus*, the agromyzid fly *Phytoliriomyza ornata* and the ephydrid fly *Hydrellia concolor*. All are reported as monophagous on flowering rush. According to information available in the literature (Häfliger et al. 2014), the authors can expect the highest impact on flowering rush through larval feeding of the weevils followed by that of the flies.

2.2 Objectives

The objectives of this research were to find the four most promising potential biological control agents, to study their biology, and to establish rearing colonies of at least two species. The aim was also to continue no-choice host-specificity tests with the weevil *B. nodulosus*.

2.3 Approach

METHODS: A total of ten sites were visited each in 2014 and 2015 (Figure 4 and Table 4). In 2014, the team made three visits (16–17 May, 5–6 August and 15–16 September) to the two sites in northern Germany (Bremen and Kiel areas), where they had found the weevil *B. nodulosus* in 2013 (Häfliger et al. 2014). In addition, the team conducted one field survey to sites in Hungary, the Slovak Republic, and Poland between 31 May and 4 June. In 2015, they made two visits to northern Germany (14–15 May Bremen and Kiel, 16 August only Kiel). Additional sites were visited in the Czech and Slovak Republics (28 May–1 June, 30 June only Slovak Republic) and Serbia (6–7 June). The authors' colleague, Sonja Stutz, kindly brought plant samples from Georgia (5 June). For some sites permits were needed, because the sites are located within nature reserves. The primary aim of these field trips was to collect more *B. nodulosus* and to search for *B. validus*. The team also wanted to collect more information about the biology and feeding habits of the two fly species *P. ornata* and *H. concolor*. At each site, plants were non-destructively searched for at least 20 minutes and any

insects found were collected with an aspirator and taken back to CABI for further investigations and/or identification. At sites with a sufficient number of flowering rush, plant samples were taken and dissected for immature insect stages in the lab. Attempts were made to rear any immatures found through to adulthood so they could be sent to specialists for identification. Feeding marks, mines, and head capsules found during dissections were also recorded and measured.

Figure 4. Location of flowering rush sites visited in 2013 (blue), 2014 (red) and 2015 (green).

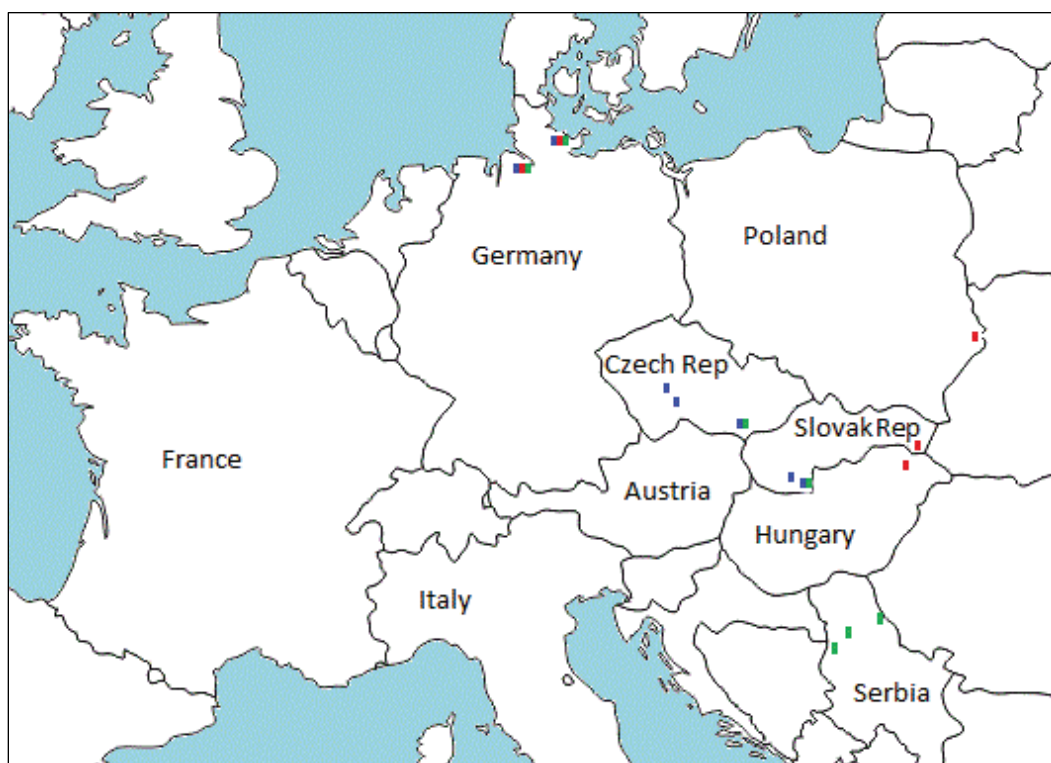


Table 4. *Butomus umbellatus* sites sampled in 2014 and 2015.

Country	Name	Ploidy ^c	Description	<i>Bagous</i> ^d	<i>Phytolirio-myza</i> ^d	<i>Hydrellia</i> ^d
Germany	Kasse-Teiche ^{a,b}	?	Fish ponds	+++	++	+
Germany	Niedervieland ^{a,b}	?	Channels	+++	+	
Hungary	Sárospatak ^a	?	Oxbow lake	++	+	
Hungary	Bálványos ^a	?	Channel	+		
Slovak Rep.	Sirník ^a	3×	Channel	+++		
Slovak Rep.	Vinička ^a	?	Channel	+		
Slovak Rep.	Bot'any ^a	3×	Oxbow lake			
Slovak Rep.	Svätá Mária ^a	3×	Channel	+		
Slovak Rep.	Vojany ^a	2×	Meadow			
Slovak Rep.	Nána ^b	?	Pond	+++ ^e	+	+

Country	Name	Ploidy ^c	Description	<i>Bagous</i> ^d	<i>Phytolirio-myza</i> ^d	<i>Hydrellia</i> ^d
Slovak Rep.	Kamenín ^b	?	Pond	+		
Czech Rep.	Lanžhot ^b	3×	Channel	+		
Czech Rep.	Hlohovec ^b	3×	Fish pond	++		
Czech Rep.	Lednice ^b	3×	Channel, pond	+		
Poland	Grodek ^a	?	Pond	++	+	
Serbia	Ševarice ^b	?	Channel	++		
Serbia	Jarkovac ^b	?	Pond	++		
Serbia	Plandište ^b	?	Channel	++	+	

^a visited in 2014

^b visited in 2015

^c 3× = triploid, 2× = diploid, ? = not yet known

^d +++ = many, ++ = several, + = few individuals

^e both *B. nodulosus* and *B. validus*

At each of six sites, ten additional leaf samples were collected and placed in silica gel for molecular analysis. The team also took seven leaf samples from plants bought at a nursery and seven from plants grown from purchased seeds. All samples were sent to Dr. John Gaskin (USDA-ARS Sidney, Montana) for molecular analysis.

RESULTS: At most sites visited, flowering rush was triploid (Krahulcová and Jarolímová 1993; Kirschner et al. 2004). The ploidy level of several sites still needs to be determined. Preliminary molecular analysis of leaf samples did not show any match yet between North American and European populations of flowering rush (J. Gaskin, pers. communication¹). Samples collected in 2015 (including plants from European nurseries) were shipped to Dr. Gaskin and will be analyzed later in the year.

Bagous nodulosus was found at 16 sites (Table 4). As far as analyzed, these were all on triploid plants. In each year, the team collected about 220 *B. nodulosus* adults to establish a rearing colony and set up host-specificity tests. In 2015, the team finally found one site with *B. validus*, the second weevil known from *B. umbellatus*. Unfortunately, the team did not find enough fly larvae and pupae to be able to start working with the two fly species.

¹ John Gaskin, USDA-ARS Sidney, Montana

2.4 *Bagous nodulosus* GYLLENHAL (Coleoptera, Curculionidae)

2.4.1 Biology

Although the weevil *B. nodulosus* (Figure 5) is considered to be a rare species in most regions (Dieckmann 1983; Gosik 2006), the team found it with no difficulty on their first field trip to northern Germany in 2013, and then later also in the Czech and Slovak Republics, Hungary, Poland and Serbia. Its preferred habitats are shallow, clear, and sun-exposed ponds or channels.

Figure 5. Adult *Bagous nodulosus*.



Females oviposit into the leaves in May and June and larvae develop within a month to adults (Gosik 2006; Häfliger et al. 2014). The weevils overwinter as adults on plant debris below-water and mate and oviposit the following spring.

It seems that timing of surveys is quite important for finding *B. nodulosus*. Observations made in the team's rearing colony and in the field suggest that the weevils spend most of their time underwater. On warm days in spring (May/June), adults can be found in large numbers feeding on emergent leaves of flowering rush. In mid-May 2014, the team collected 120 adults at the two sites in northern Germany, and these represented less than half of the weevils observed in a subset of the *Butomus* stands. Team members did not want to collect more adults, because one of the sites is protected and there are still concerns about the weevil being endangered. In August, only three adults were found at the same sites, and

none in September. However, in spring 2015, a large number of adults were again observed.

A minimum level of water appears to be important for the weevils. One of the *Butomus* sites in a wet meadow in northern Germany was almost completely dried up between May and August 2014, and no weevils were found during this period. However, in September 2014, when the meadow was covered again with about 10 cm of water, the team was able to collect 26 weevils that were sitting on the leaves 1–3 cm below the surface of the water. The observations made in 2014 confirm the authors' earlier assumption that *B. nodulosus* spends most of its life underwater and the frequency and occurrence of the species is therefore often underestimated.

2.4.2 Differentiation of sexes

INTRODUCTION: At the beginning of the project, there were difficulties differentiating males from females of *B. nodulosus*. It is known from the literature that the rostrum of *B. validus*, a sibling species to *B. nodulosus*, is longer in females than in males (Dieckmann 1983). Indeed, it was possible, with some experience, to use this characteristic to identify *B. nodulosus* weevils with longer and more slender rostrums as females and those with shorter and stouter rostrums as males (see Figure 6). However, rostrum length alone was not sufficient in all cases to clearly separate male and female *B. nodulosus*.

Figure 6. Male *Bagous nodulosus* sitting on the back of a female (rostrum measurements taken for sexing marked in red and blue).



METHODS: About 20 weevils that died during rearing were dissected to confirm gender. In addition, the team took a number of measurements of seven males and eight females; i.e., length of rostrum from the point of insertion of the antenna to the eye and to the tip, width of rostrum, distance between the eyes, width of the head, etc., and calculated various ratios.

RESULTS: The mean length of the rostrum was significantly shorter for males than females ($P = 0.004$). However, the ratio of rostrum width between points of insertion of antennae (red on Figure 6) and length of rostrum from point of insertion of antenna to the tip (blue on Plate 3) was an even more reliable characteristic to separate males (>0.846) from females (<0.846) (Table 5). Team members successfully used this criterion on 15 unsexed weevils and are confident that they developed a reliable technique for distinguishing males from females.

Table 5. Measurements of rostrum for sexing *Bagous nodulosus*.

Sex	Width of rostrum (mm)	Length of rostrum from insertion of antenna to tip (mm)	Ratio width:length of rostrum
Female	0.247	0.420	0.588
Female	0.272	0.445	0.611
Female	0.296	0.469	0.632
Female	0.296	0.420	0.706
Female	0.296	0.395	0.750
Female	0.296	0.371	0.800
Female	0.346	0.420	0.824
Female	0.272	0.321	0.846
Mean \pm SE	0.290 \pm 0.01	0.408 \pm 0.02	0.720 \pm 0.04
Male	0.272	0.321	0.846
Male	0.321	0.371	0.867
Male	0.321	0.371	0.867
Male	0.272	0.296	0.917
Male	0.296	0.321	0.923
Male	0.296	0.296	1.000
Male	0.247	0.420	0.588
Mean \pm SE	0.296 \pm 0.01	0.329 \pm 0.01	0.903 \pm 0.03

2.4.3 Rearing

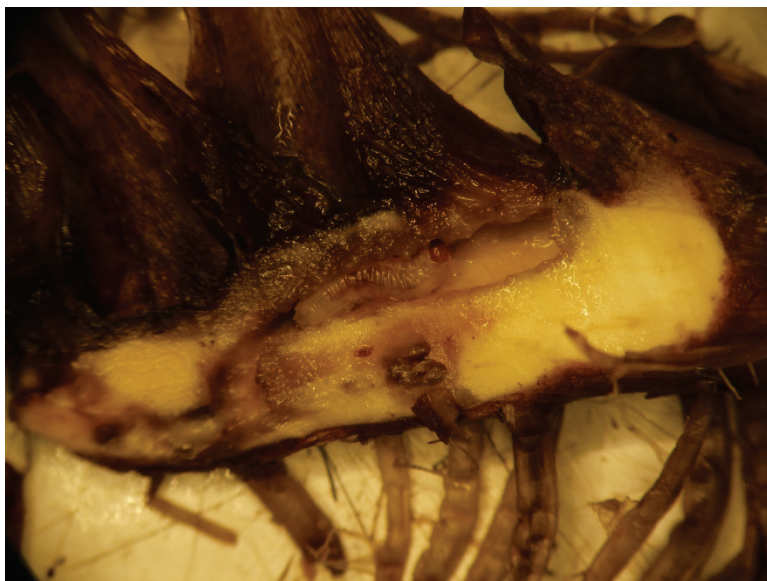
METHODS: In order to study the biology of *B. nodulosus*, and to be less dependent on field collections for host-specificity tests, a rearing colony was established. Overwintered or field-collected adults were kept in cylinders and provided with cut leaves of flowering rush. Leaves were replaced after 3–5 days and dissected for eggs before disposal. Eggs found were kept in Petri dishes (diameter 5.5 cm) on moist filter paper until larval hatch. More than 900 newly emerged larvae were transferred between mid-May and early August 2014 onto about 70 potted flowering rush plants covered with gauze bags. An additional 66 adults were kept for oviposition on 17 potted plants covered with gauze bags for 1–3 weeks. In 2015, 270 adults were set up on 60 potted plants for oviposition. Plants were grown in 2-liter pots (height: 17 cm, diameter: 14 cm) and mostly separated and repotted in spring. Plants were kept submerged to a depth of 3–20 cm in two 4 m × 2 m pools. In 2015, the team transferred 230 larvae on 50 potted plants and 150 larvae on rhizome pieces wrapped in tissue paper. In 2015, most of the plants were not kept submerged; rather, they were in trays filled with a few centimetres of water. Plants were dissected for larvae or pupae after 2–4 weeks. Some plants were only checked for emerged adults after 6–8 weeks. Whenever possible, head capsule diameters were measured to determine the larval instar. In 2015, about half of the plants were not checked for emerging adults. This will be done in spring 2016.

In an artificial pond, the team overwintered about 60 adults on 12 potted plants covered with gauze bags in 2014/2015. An additional 20 pots, onto which larvae had been transferred but no adults had emerged, were overwintered in the same way, since there is some potential that individuals were overlooked. About 100 adults were set up in fall 2014, like the winter before, in stacked plastic cylinders (height: 27 cm, diameter: 11 cm) filled to a depth of 4–8 cm with water. In fall 2015, the team set up about 180 *B. nodulosus* on 30 potted plants for overwintering in an artificial pond.

RESULTS: Weevils collected in mid-May 2014 in northern Germany had stopped ovipositing by the end of May, while weevils collected in June in the Slovak Republic and Hungary continued ovipositing until the end of July. In 2015, the team observed the same pattern; i.e., German weevils stopped ovipositing much earlier than weevils from the Slovak Republic and Serbia. There is as of yet no explanation for this difference between the populations.

Most of the larvae transferred onto potted plants died before or immediately after pupation. The team is unsure why this happened, but hypothesize that it might be related to water temperature/quality, bacterial infection or predation. Different testing set-ups were attempted, keeping plants in shallow water, deep water, unsubmerged, or in buckets with regularly exchanged water. Keeping the plants unsubmerged slightly increased rearing success, but the highest survival rate from first instar larva to adult was obtained by transferring larvae onto rhizome pieces wrapped in tissue paper. From 150 larvae transferred, 30 adults emerged (Figure 7). However, these adults were distinctly smaller than adults emerging from potted plants.

Figure 7. Flowering rush rhizome damaged by *B. nodulosus* larva.



Mortality of weevils overwintering in cylinders in a wooden shelter was quite high. From 108 adults set up in October 2014, only 40 survived until March 2015. However, the team experienced an extremely high survival rate of 80% for adults overwintering on potted plants covered with gauze bags in an artificial pond.

2.4.4 Host-specificity tests

From mid-May to mid-June 2014, and from early June to mid-August 2015, the team established the first host-range trials, set up as sequential no-choice oviposition tests. Since it would require too much material in terms of plants and insects, the team did not use potted plants, but tested a set-up using cut leaves instead.

METHODS: To ensure that only egg-laying females were used for this test, females were kept individually for 1–2 days on a *Butomus* leaf. Only females that laid at least one egg within two days were used. Cut leaves of test plants were individually exposed to ovipositing females for two days in plastic cylinders (volume 1.3 litres) half-filled with water. Females were then placed onto cut leaves of flowering rush to verify that they were still laying eggs. Tests were only considered valid if the female laid at least one egg on the control (flowering rush) within three days after the test. Females that were still laying eggs were subsequently exposed to another set of test plants. Eggs found during the tests were used to supplement the rearing colony.

RESULTS: Using this method, a total of 22 test plant species, 14 native to North America, were exposed to *B. nodulosus* females in 2014 and 2015. A few feeding marks were found on some of the test plants, but none of them were accepted for egg laying (Table 6).

2.4.5 Molecular analysis

Since literature records indicated that *B. nodulosus* from Poland are larger, and because this could imply genetic differentiation, the team decided to analyze the different weevil populations using molecular methods.

Molecular analysis of all *B. nodulosus* populations sampled in 2014 was carried out by a colleague, Ivo Toševski (Institute for Plant Protection and Environment, Zemun, Serbia). Samples collected in 2015 will be analyzed during winter 2015/2016. The relevant barcoding region of the genus *Bagous* turned out to be very difficult to amplify. This could explain why DNA sequences from *Bagous* species are absent from the public GeneBank database and also from the BOLD database (Barcoding of Life). The analysis showed only minor genetic divergence between geographically distant populations. *Bagous nodulosus*, therefore, appears to be a very compact species with low genetic variation (Figure 8). This is useful background information, if one decides to work with different *B. nodulosus* populations.

Table 6. Results of sequential oviposition tests with *Bagous nodulosus* in 2014 (red) and 2015 (black).

Plant species	# replicates set up	# replica-tes valid	# eggs	feeding ^b
<i>Butomus umbellatus</i>	399+650		529+281	+++
<i>Butomus umbellatus (american)</i>	84		115	+++
<i>Alisma plantago-aquatica</i>	5	3	0	+
<i>Alisma triviale</i> ^a	11	5	0	-
<i>Blyxa aubertii</i>	3	2	0	++
<i>Ceratophyllum demersum</i> ^a	5	1	0	+
<i>Elodea canadensis</i> ^a	7+8	4+1	0	+
<i>Elodea densa</i>	6	5	0	+
<i>Carex obnupta</i> ^a	9	4	0	-
<i>Echinodorus berteroi</i> ^a	9	4	0	++
<i>Echinodorus cordifolius</i> ^a	8	2	0	+
<i>Hydrilla verticillata</i>	8+3	5+1	0	+
<i>Hydrocharis morsus-ranae</i>	6	3	0	+
<i>Myriophyllum spicatum</i>	5+8	3+4	0	++
<i>Nuphar lutea</i> ^a	12	4	0	-
<i>Polygonum amphibium</i> ^a	5	3	0	+
<i>Potamogeton natans</i>	6	2	0	+
<i>Potamogeton lucens</i>	4	1	0	+
<i>Schoenoplectus acutus</i> ^a	8	5	0	-
<i>Schoenoplectus americanus</i> ^a	2	1	0	+
<i>Schoenoplectus tabernaemontani</i> ^a	8	5	0	+
<i>Sagittaria graminea</i> ^a	6+6	3+3	0	+
<i>Sagittaria latifolia</i> ^a	5+10	3+2	0	+
<i>Sagittaria platyphylla</i> ^a	7+2	6+1	0	+

^a native to North America^b - = no feeding, + = minor feeding on single leaves, ++ = some feeding on few leaves, +++ = major feeding on most leaves

Figure 8. Evolutionary relationships of 24 taxa: the evolutionary history was inferred using the neighbor-joining method (p-distance model), comparing 613 nucleotide positions in the final data set from mtCOI (mitochondrial cytochrome oxidase 1) gene.



2.4.6 Impact experiment

Between 21 and 27 May 2015, an impact experiment was established by releasing different densities (0, 1, or 3 pairs) of *B. nodulosus* onto individually potted, gauze-covered flowering rush plants. The plants used had on average 27 leaves and the longest leaf was on average 78 cm long. Ten replicates were established per density. Only egg-laying females were used. Prior to setup, plant size was measured and it was ensured that there were no initial differences in plant parameters between treatments. After two weeks, weevils were removed from the plants and feeding damage quantified. At the end of July, plants were checked for emerged weevils and above- and belowground biomass recorded. However, due to the high larval mortality that was also observed during the rearing, only one weevil was found completing development and no significant impact could be

found. The team will repeat this experiment, as soon as they find a more reliable rearing method.

2.5 Other species

Based on the literature and field surveys conducted in 2013, the team prioritized three additional insects as potential biocontrol agents. All three species are recorded only from flowering rush.

2.5.1 *Bagous validus* ROSENHAUER (Coleoptera, Curculionidae)

Detailed site records for *B. validus*, a sibling species to *B. nodulosus*, are very rare. The only recently published record is from Serbia (Pesic 2002). The Hungarian entomologists Otto Merkl, Attila Podlussány and Gábor Hegyessy (Hungarian Natural History Museum, Budapest) sent the team the coordinates of a few sites in Hungary where this weevil was found about 20 years ago. However, team members found flowering rush at only one of these sites in 2014, and *B. validus* was not found there at all. Petr Bogusch (University of Hradec Králové) also showed the team a site in the Czech Republic where the species was found a few years ago. Again, *B. validus* could not be found either, when the site was visited in 2015.

Team members finally found one site in Southern Slovakia in 2015, where they were able to collect 25 adults. Last records of *B. validus* in this region are more than 30 years old (Dieckmann 1983). Like Dieckmann, the team found both *Bagous* species co-occurring. Although 12 females were collected, only 6 eggs were obtained during the summer (Figure 9). These eggs were not found in holes in the leaves like *B. nodulosus*, but on the surface of the leaves or of the rearing container. This was also observed by Dieckmann (1983). However, the fact that only a few eggs were found likely indicates that the species has special requirements for oviposition that the team was unable to provide.

Half of the six larvae obtained in the rearing were transferred onto potted plants, half onto rhizomes wrapped in tissue paper. One larva completed development to adult on a rhizome piece. Nine pairs are now being overwintered in the authors' artificial pond on 4 potted plants covered with gauze bags.

Figure 9. *Bagous validus* females (with egg on the right).



2.5.2 *Phytoliriomyza ornata* (MEIGEN) (Diptera, Agromyziada)

The genus name of this agromyzid fly (syn. *Metopomyza ornata*) has been changed several times in the past. According to the agromyzid specialist Prof. von Tschirnhaus (Bielefeld, Germany), the current valid genus name *Phytoliriomyza* should be changed back to *Cerodontha*.

The team found about 20 pupae of *P. ornata* upon dissection of flowering rush leaves collected in northern Germany in August and September 2014 (Figure 10). However, apart from a few parasitoids, only one fly emerged in fall 2014 and one after overwintering in spring 2015. The team found several additional larvae and pupae during dissections of field-collected plants in 2015. However, it was not possible to obtain eggs and establish a rearing colony. The team will try to obtain more flies in 2016 by visiting more sites and setting up adult emergence traps with field-collected material of flowering rush.

2.5.3 *Hydrellia concolor* (STENHAMMER) (Diptera, Ephydriidae)

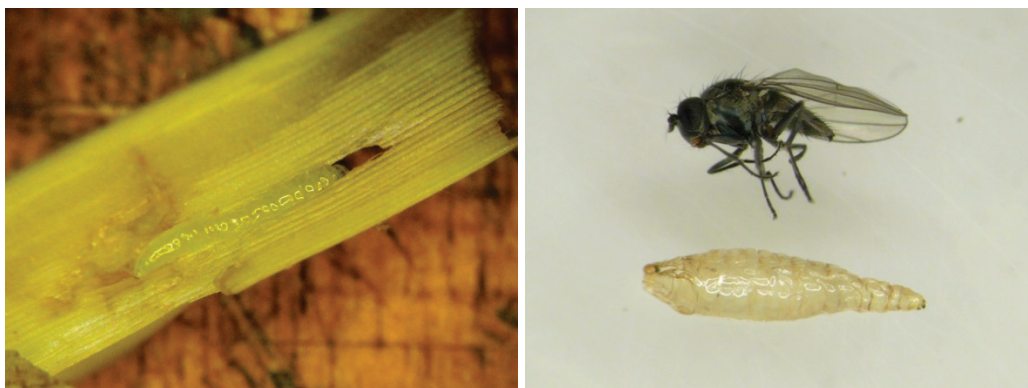
The only four specimens of an ephydrid fly that the team was able to rear through or collect as adults in 2013 were sent for identification to Jens-Hermann Stuke (Leer, Germany) (Figure 11). Since they were all females, and males are necessary for the identification of species, it was only possible to confirm that they belong to the genus *Hydrellia*. Although there is only one *Hydrellia* species recorded on flowering rush in the literature, the team has found two different species of this genus. Unfortunately, it was only possible to collect empty *Hydrellia* pupal cases in 2014 and 2015. The team will need to collect flowering rush earlier in 2016 in order to obtain a

sufficient number of males for definite identifications. As for *P. ornata*, the team hopes to be more successful by using emergence traps instead of labor-intensive dissection of plants.

Figure 10. Pupae and adult of the agromyzid fly *Phytoliriomyza ornata*.



Figure 11. Larva and adult *Hydrellia* sp. with empty pupal case.



2.6 Test plants

In collaboration with their North American partners, the team started developing a test plant list for host-specificity testing (for details see section 6 in the 2013 annual report (Häfliger et al. 2014). A total of 48 taxa are currently included in the list. The final number tested will depend on the availability of test plant material and the team's ability to grow plants under artificial conditions. Currently, they are successfully growing 28 test plant species (ten European and 18 native North American species). The native North American species were provided by Jenifer Parson (Washington Department of Ecology) and Peter Rice (University of Montana, Missoula) mostly as rhizomes or tubers. Some species were grown from cold- and wet-

stratified seeds. In addition, the team is growing flowering rush plants of seven U.S. populations to assure that potential agents will attack the flowering rush genotypes present in North America.

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14. ABSTRACT The noctuid moths, <i>Archana geminipuncta</i> and <i>A. neurica</i> , were selected as the most promising candidates for biological control of common reed. Successful larval development was only found on <i>Phragmites</i> spp., but development was also possible on the native North American subspecies <i>P. australis</i> subsp. <i>americanus</i> . However, open-field oviposition tests showed a strong preference of females for both European and introduced <i>P. australis</i> . Because of the higher egg mortality on native reed, the authors expect any impact of <i>A. neurica</i> and <i>A. geminipuncta</i> on the native reed <i>P. australis</i> subsp. <i>americanus</i> to be negligible, should the noctuids be released in North America. A biological control project for flowering rush was started in spring 2013. According to the authors' literature survey, the semi-aquatic weevil, <i>Bagous nodulosus</i> , is the most promising potential agent for biological control. During various field surveys in Northern Germany, the Czech and Slovak Republics, Hungary, Poland, and Serbia, the authors collected several hundred adults of <i>B. nodulosus</i> , established a rearing colony, and started with sequential no-choice oviposition tests. So far, no eggs were found on any of the 22 test plants offered. The authors also found the other five herbivore species recorded as monophagous on flowering rush and started work with a second weevil, <i>B. validus</i> .					
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